REMARKS

Claims 1-22, 25 and 26 are pending in the subject application. The Examiner has withdrawn claims 6-8, 20-22 and 25-26 as being drawn to a nonelected invention. Applicants have hereinabove amended claims 1, 5, 15, and 19, added new claim 27 and canceled claims 25 and 26 without disclaimer or prejudice to applicants' right to pursue the subject matter of these claims in the future. Support for the amendments to the claims may be found, *inter alia*, in the specification as follows: claim 1: page 10, lines 19-29; claim 5: page 10, liens 19-21; claim 15: page 11, lines 21-32; and claim 19: page 11, lines 21-23. Support for new claim 27 may be found in the specification as follows: page 10, lines 3-10 and 19-21. Upon entry of this Amendment, claims 1-22 as amended and new claim 27 will be pending and claims 1-3, 5, 9-17, 19, as amended, and new claim 27 will be under examination.

Group Art Unit

Applicants acknowledge that the Group Art Unit of the subject application has changed and will direct all correspondence regarding this application to Examiner Gregory S. Emch in Art Unit 1649.

Elections/Restrictions

The Examiner acknowledged on page 2 of the April 27, 2009 Office Action applicants' election of Group I, claims 1-5 and 9-19 and of the species of cell death in the hippocampus. The Examiner withdrew the election of species requirement set forth in the December 3, 2008 Office Action on the grounds that examining all the species together does not represent a tremendous search burden. The Examiner indicated that claims 6-8, 20-22, and 25-26 are withdrawn from further consideration pursuant to 37 CFR §1.142(b) as being drawn to a nonelected invention, there being no allowable generic or linking claim. The Examiner further indicated that claims 1-5 and 9-19 are under Examination in the instant office action.

Applicants note that the cover page of the April 27, 2009 Office Action lists claims 4 and 18 as being withdrawn from consideration.

However, as noted above, the Examiner withdrew the election of species requirement and also indicated that claims 1-5 and 9-19 are under Examination. Accordingly, applicants note that claim 4 and 18 are listed as "withdrawn" in the listing of claims which begins on page 2 of this paper. Applicants respectfully request that the Examiner indicate that these claims are no longer withdrawn.

Rejections Under 35 U.S.C. §112, first paragraph

The Examiner rejected claims 1-4 and 9-18 under 35 U.S.C. §112, first paragraph, as allegedly failing to comply with the written description requirement. The Examiner asserted that the claims contain subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention. Specifically, the Examiner indicated that claims 1-4 and 9-18 require the use of an inhibitor of receptor for advanced glycation endproducts (RAGE). The Examiner indicated that inhibitors of RAGE are described at page 10, lines 19-29 of the specification and that such inhibitors can include an an antibody, which when contacted with RAGE, specifically inhibits binding between RAGE and a ligand thereof, an anti-sense molecule which specifically inhibits the expression of RAGE in a cell, an RNAi molecule which specifically inhibits the expression of RAGE in a cell or a catalytic nucleic acid which specifically inhibits expression of RAGE in a cell. The Examiner asserted that the specification does not describe which amino acid residues or nucleic acid residues are present in the genus of claimed RAGE inhibitors. The Examiner asserted that the specification fails to disclose which regions of RAGE inhibitors are responsible for reducing neuronal damage. Based on the foregoing the Examiner concluded that because the specification does not disclose which amino acid and/or nucleic acid residues are common to all RAGE inhibitors and or which structures are either necessary or sufficient such that members of the genus have the required activity that the claims do not meet the written description requirement.

In response, applicants respectfully traverse the Examiner's ground of rejection. Nevertheless, without conceding the correctness of the Examiner's rejection, applicants have hereinabove amended claim 1.

Claim 1, as amended hereinabove, recites in relevant part "...wherein the inhibitor of RAGE is selected from the group consisting of an antibody, an antisense molecule, an RNAi molecule, and a catalytic nucleic acid.

Applicants note that the sequence of the RAGE gene was known in the art, as disclosed, *inter alia*, on page 8, lines 20-27 of the specification and as evidenced by Neeper et al. (1992), attached hereto as **Exhibit A**, which describes the nucleotide and amino acid sequences of bovine and human RAGE. In addition, applicants note that methods of making and producing antibodies, antisense molecules, RNAi molecules and catalytic nucleic acids were known in the art at the time of filing the subject application. As noted by the Examiner on pages 3 and 4 of the April 27, 2009 Office Action, the subject specification discloses that the RAGE inhibitors may be (a) an antibody which, when contacted with RAGE, specification inhibits binding between RAGE and a ligand thereof, (b) an anti-sense molecule which specifically inhibits the expression of RAGE in a cell, (c) an RNAi molecule which specifically inhibits the expression of RAGE in a cell and (d) a catalytic nucleic acid which specifically inhibits the expression of RAGE in a cell.

Accordingly, applicants maintain that amended claim 1 and the claims dependent therefrom satisfy the written description requirement of 35 U.S.C. §112, first paragraph.

In view of the preceding remarks, applicant's respectfully request that the Examiner reconsider and withdraw this ground of rejection under 35, U.S.C. §112, first paragraph.

Rejections Under 35 U.S.C. §103

The Examiner rejected claims 105 and 9-19 under 35 U.S.C. §103(a) as allegedly unpatentable over Ganesh et al. (Hum. Mol. Genet. (2002)); in view of Yan et al. (Nature 1996), further in view of Lado et al. (Epileptic Disord. 2002). The Examiner asserted that it would have been prima facie obvious to the person of ordinary skill in the art at the time of the invention to arrive at the claimed invention by combining the disclosures of Ganesh et al., Yan et al. and Lado et al. Specifically, Examiner asserted that in view of Ganesh et al., the skilled artisan would have know that AGEs are associated with neuronal damage in the hippocampus and that inhibiting binding of AGE and RAGE to reduce the neuronal damage would be desirable for treating subject with seizure disorders. The Examiner further asserted that in view of Yan et al., the skilled artisan would have known that ligand-RAGE binding is implicated in the neuronal damage associated with Alzheimer's disease and that treatment of a subject with an antibody which inhibits ligand-RAGE binding to be effective in reducing neuronal damage. The Examiner also asserted that in view of Lado, the skilled artisan would have known that hippocampal neuronal damage can occur immediately after the first seizure in a human and that early aggressive therapy for seizures is desirable. Based on the foregoing, the Examiner it would be reasonable to predict that that RAGE blocking antibody of Yan et al. could be successfully used to reduce the neuronal damage resulting from seizures, asserting that Yan's results with Alzheimer's disease related pathology supports a reasonable expectation of success.

In response, applicants respectfully traverse the Examiner's ground of rejection.

Ganesh et al. disclose that in a mouse model for *Lafora disease* advanced glycation endproducts are present in neuronal Lafora bodies (emphasis added). Ganesh et al. also disclose that Lafora inclusions may induce neuronal stress and neurotoxicity due to a diminished ubiquitin proteolytic system and/or by generation of reactive oxygen species through an interaction between AGE and RAGE (see paragraph

bridging page 1259 to 1260). Nowhere do Ganesh et al. disclose administering a RAGE inhibitor to reduce the extent of neuronal damage which would otherwise result from a seizure. In fact, Ganesh et al. make no disclosure of any RAGE inhibitors.

Yan et al. disclose that amyloid-beta peptide is a RAGE ligand and further disclose that blocking this interaction with an anti-RAGE IgG or soluble RAGE inhibits binding between amyloid-beta peptide and RAGE (see page 687). Yan et al. disclose that the amyloid-beta peptide-RAGE interaction may contribute to neurotoxicity that results in *dementia.* (emphasis added) Yan et al. do not disclose or suggest administering a RAGE inhibitor to reduce the extent of neuronal damage which would otherwise result from a *seizure* (emphasis added). Yan et al. do not disclose any antisense molecule, RNAi molecule, or catalytic nucleic acids which are RAGE inhibitors.

Lado et al. disclose that a first seizure may produce hippocampal injury and synaptic rearrangement and that some would favor early aggressive intervention to prevent the cycle of seizures from producing additional proconvulsant injury to the brain.

Applicants maintain that no combination of Ganesh et al. with Yan et al. and Lado et al. would render obvious applicant's claimed invention. Specifically, applicants note that none of the references disclose or suggest that neuronal damage which would otherwise result from a seizure can actually be reduced by administering a RAGE inhibitor, as recited in applicants' amended claim 1. The Examiner asserted on page 8 of the April 27, 2009 Office Action that all of the references teach disorders that involve neuronal damage resulting from cellular dysfunction and cell death in the hippocampus and cerebral cortex. However, the Examiner has provided no evidence to show that a person skilled in the art would have a reasonable expectation that compounds which may be desirable for treating amyloid-beta mediated neuronal damage in Alzheimer's disease (as disclosed in Yan et al.) may also be successful in treating neuronal damage which would otherwise result from a seizure. As such,

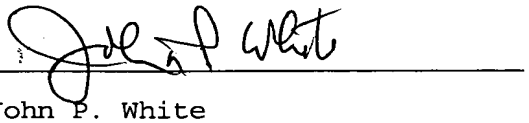
applicants maintain that the combination of Ganesh et al. with Yan et al. and Lado et al. does not render obvious applicant's claimed invention.

In view of the preceding remarks, applicants respectfully request that the Examiner reconsider and withdraw this ground of rejection under 35 U.S.C. §103

If a telephone interview would be of assistance in advancing prosecution of the subject application, applicants' undersigned attorney invites the Examiner to telephone him at the number provided below.

No fee, other than the enclosed $490.00 fee for a two-month extension of time, is deemed necessary in connection with the filing of this Amendment. If any additional fee is required, authorization is hereby given to charge the amount of any such fee to Deposit Account No. 03-3125.

Respectfully submitted,



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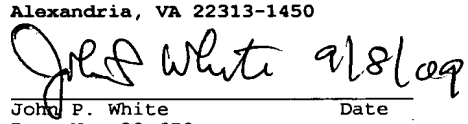

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EXHIBIT A

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Cloning and Expression of a Cell Surface Receptor for Advanced Glycosylation End Products of Proteins*

(Received for publication, January 23, 1992)

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Advanced glycosylation end products of proteins (AGEs) are nonenzymatically glycosylated proteins which accumulate in vascular tissue in aging and at an accelerated rate in diabetes. A ~35-kDa polypeptide with a unique NH_2-terminal sequence has been isolated from bovine lung and found to be present on the surface of endothelial cells where it mediates the binding of AGEs (receptor for advanced glycosylation end product or RAGE). Using an oligonucleotide probe based on the amino-terminal sequence of RAGE, an apparently full-length cDNA of 1.5 kilobases was isolated from a bovine lung cDNA library. This cDNA encoded a 394 amino acid mature protein comprised of the following putative domains: an extracellular domain of 332 amino acids, a single hydrophobic membrane spanning domain of 19 amino acids, and a carboxyl-terminal domain of 43 amino acids. A partial clone encoding the human counterpart of RAGE, isolated from a human lung library, was found to be ~90% homologous to the bovine molecule. Based on computer analysis of the amino acid sequence of RAGE and comparison with databases, RAGE is a new member of the immunoglobulin superfamily of cell surface molecules and shares significant homology with MUC 18, NCAM, and the cytoplasmic domain of CD20. Expression of the RAGE cDNA in 293 cells allowed them to bind ^{125}I-AGE-albumin in a saturable and dose-dependent manner (K_d ~100 nM), blocked by antibody to RAGE. Western blots of 293 cells transfected with RAGE cDNA probed with anti-RAGE IgG demonstrated expression of immunoreactive protein compared to its absence in mock-transfected cells. These results suggest that RAGE functions as a cell surface receptor for AGEs, which could potentially mediate cellular effects of this class of glycosylated proteins.

Advanced glycosylation end products of proteins (AGEs)[1] result from the prolonged exposure of proteins to aldoses, such as glucose and ribose, and have been shown to be present in the plasma and to accumulate in tissues at an accelerated rate in diabetes (1–5). Although the AGEs are a heterogeneous class of compounds, their ability to form cross-links to and between proteins, and their interaction with a class of binding sites on endothelial cells and monocytes (6–9), as well as other cell types (10), suggests two mechanisms through which they could contribute to diabetic complications: by altering the architecture of the extracellular matrix through the formation of cross-links between basement membrane components (1), and by modulating cellular function following interaction with cell surface binding sites.

AGEs perturb a broad range of cellular functions, especially in endothelial cells and macrophages (6–8). For example, in cultured endothelium AGEs increase permeability and expression of procoagulant activity, and AGEs induce migration of mononuclear phagocytes, as well as production of platelet-derived growth factor and cytokines (10, 11). These considerations led us to characterize surface receptors potentially mediating the cellular interactions of AGEs. We have isolated a 35-kDa polypeptide with an unique NH_2-terminal sequence which is present on the endothelial cell surface and binds AGEs selectively and saturably (7). In this study, we report the cloning and expression of this receptor for advanced glycosylation end products (RAGE). The results indicate that RAGE is a new member of the immunoglobulin superfamily of receptors. Expression of the RAGE cDNA in 293 cells resulted in detection of RAGE antigen on the cell surface, and the ability of the cells to bind ^{125}I-AGE albumin. These data, indicating that RAGE is a cell surface receptor which can interact with AGEs, form the basis for future studies examining the mechanisms underlying cellular effects of this class of glycosylated proteins.

MATERIALS AND METHODS

Isolation of the 35-kDa AGE Receptor, Trypsin Cleavage, and Sequencing of Peptides—The 35-kDa AGE receptor (RAGE) was purified as described previously (7): acetone extract of bovine lung was subjected to chromatography on hydroxylapatite, fast protein liquid chromatography Mono S, and gel filtration. RAGE was homogenous on reduced and nonreduced SDS-PAGE. Purified RAGE was subjected to tryptic digestion in ammonium bicarbonate (0.2 M) at an enzyme-to-substrate ratio of 1:30 (w/w) at 37 °C for 20 h. HPLC

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) M91211 and M91212.

¶ Recipient of a fellowship award from the Juvenile Diabetes Foundation.

‡‡ Completed this work during the tenure of a Genentech-EI Award from the American Heart Association.

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[1] The abbreviations used are: AGEs, advanced glycosylation end products; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC high performance liquid chromatography; bp, base pair(s).

peptide mapping of the tryptic digest was performed with a Hewlett-Packard 1090 System (Avondale, PA) equipped with a 2 × 150-mm reversed-phase C-8 column (Phase Separation Inc., Norwalk, CT). Peptides were eluted with an acetonitrile gradient in trifluoroacetic acid (0.1%), and peptide-containing fractions were collected for further analysis. For the reduced peptide map, the digest was treated with 2-mercaptoethanol at 100 °C. As indicated, peaks from the HPLC column were subjected to sequence analysis using an Applied Biosystems gas-phase sequencer (model 470A, Foster City, CA). Phenylthiohydantonin amino acid derivatives were identified "on-line" with an ABI model 120 phyenylthiohydantonin analyzer.

Isolation of Bovine cDNA for the 35-kDa AGE Receptor (Bovine RAGE)—A cDNA library from λgt11 made from bovine lung mRNA (Clontech, Palo Alto, CA) was screened with a synthetic oligonucleotide probe prepared on a 380A DNA Synthesizer (Applied Biosystems, Foster City, CA) of sequence 5′-AACTGCAAGGGCGCCC-CCAAGAAGCCCCCCCAGCAG-3′ based on back-translation of the amino-terminal protein sequence Asn-*X*-Lys-Gly-Ala-Pro-Lys-Lys-Pro-Pro-Gln-Gln where *X* was assumed to be cysteine. Codon choices were made by the method of Lathe (12). Lifts were prepared and hybridized in 6 × SSC, 10 × Denhardts solution, 50 mM sodium phosphate, pH 7.0, 0.1% sodium dodecyl sulfate, and 50 μg/ml yeast tRNA (18). Positive plaques were picked and purified, and inserts were subcloned into pUC19 for DNA sequencing.

DNA Sequencing and Database Search—The DNA sequence was determined by the dideoxy chain termination method (13) using Sequenase as recommended by the manufacturer (U. S. Biochemical, Cleveland, OH). Areas of secondary structure were resolved in parallel sequencing reactions employing dITP as specified by the manufacturer. Initial sequences were generated at both ends of the bovine cDNA using pUC vector primers. Based on this information, DNA primers were prepared and used in sequencing reactions to extend the read. This cycle was repeated until the clone was bridged, and then additional oligomers were prepared such that each base was sequenced at least twice, most in both directions. A similar strategy was employed for the human RAGE cDNA except that many of the bovine primers were used for sequencing of the human template. Database searches of Genbank, EMBL, and SwissProt were run using the FastA, TFastA, WordSearch, and ProFile Search programs available from Genetics Computer Group (GCG) (14). The hydrophilicity profile was obtained using the Hopp and Woods program from Intelligenetics Corporation (Mountain View, CA). Determination of signal sequence cleavage sites were predicted by the Sigcleave program (29).

Northern Analysis of Bovine Lung RNA—Total RNA was isolated from adult bovine lung tissue using the guanidinium thiocyanate method (18). 25 μg of RNA was separated on a formaldehyde gel and transferred to a nylon membrane (ICN, Irvine, CA) employing methods as described (18). The blot was hybridized with the bovine cDNA for RAGE under the conditions utilized in the cloning of the human cDNA below. The RNA molecular weight markers (size range 0.24–9.5 kilobases) were obtained from Bethesda Research Laboratories.

Isolation of Human cDNA for RAGE—A cDNA library from λgt11 made from human lung mRNA (Clontech) was screened with the full-length bovine RAGE cDNA ^{32}P-labeled by a random priming reaction according to the manufacturer's instructions (Boehringer Mannheim). Positive clones were picked, purified, and subcloned in pUC19 for sequencing as for the bovine molecule above. The hybridization conditions were: 500 mM NaCl, 250 mM sodium phosphate, pH 7.2, 1 mM EDTA, 10 mg/ml bovine serum albumin, and 7% SDS at 65 °C, and the final wash temperature was 55 °C in 0.5 × SSC.

Expression Studies in 293 Cells—The bovine cDNA was released from pUC19 using *Eco*RI and inserted into the mammalian expression vector pD5 behind the adenovirus major late promoter (16) at the *Bam*HI site by a Klenow fill-in reaction (18) of insert and vector prior to ligation. A properly oriented clone was obtained (designated pD5-RAGE), and DNA was prepared for transfection studies. Calcium phosphate precipitates of pD5-RAGE DNA and pD5 DNA without an insert were used to separately transfect 293 cells (ATCC, Rockville, MD) by the method of Wigler *et al.* (15). Transient transfectants were utilized for assay at 24–48 h post-transfection. Stable lines were prepared by selecting for resistance to G418 (Geneticin, Gaithersburg, MD) with selection conditions of 250 μg/ml G418 and maintenance at 200 μg/ml.

Transfected cells were studied for expression of RAGE by assessing expression of the antigen using anti-RAGE IgG prepared from polyclonal guinea pig antiserum by chromatography on protein A-agarose (Schleicher & Schuell), and the binding of ^{125}I-AGE-albumin, prepared and radiolabeled as described previously (7).

Immunofluorescence—Immunofluorescence on nonpermeabilized 293 cells transfected with RAGE cDNA or mock-transfected 293 controls employed cell layers grown on coverslips fixed in paraformaldehyde (2%). RAGE was visualized with guinea pig anti-RAGE IgG (7) which was revealed with fluorescein isothiocyanate-conjugated goat anti-guinea pig IgG (Sigma), as described previously (20).

The presence of immunoreactive RAGE protein was also studied by Western blotting using the same anti-RAGE IgG on detergent extracts of 293 cells transfected with the cDNA for bovine RAGE or mock-transfected 293 controls (~10^8 cells in each case). 293 cells were harvested by gentle scraping, washed twice with phosphate-buffered saline, and extracted for 4 h at 4 °C in buffer containing Tris (20 mM), NaCl (0.1 M), phenylmethylsulfonyl fluoride (1 mM), trasylol (0.1%), and 1% octyl-β-glucoside, final pH 7.4. The lysate was centrifuged (11,000 × *g* for 30 min at 4 °C), and the supernatant was adsorbed to a hydroxylapatite column (bed volume, 10 ml) equilibrated in Tris (20 mM), NaCl (100 mM), and 0.1% octyl-β-glucoside, final pH 7.4. The column was washed with 10 bed volumes of equilibration buffer until the absorbance at 280 nm was <0.01, then eluted in the same buffer containing additional NaCl (1 M). The eluate was pooled and precipitated in trichloroacetic acid (20%) to precipitate the proteins. Pilot studies with purified RAGE demonstrated that this treatment did not result in degradation. The pellet was washed in ice-cold acetone three times (total of 6 ml), evaporated to dryness, and solubilized in nonreducing SDS sample buffer (21). SDS-PAGE (10%) was performed, and proteins were either visualized directly by Coomassie Blue staining or transferred electrophoretically to nitrocellulose. Excess sites on the nitrocellulose membranes were blocked using nonfat dry milk by the Blotto procedure (22), and then blots were incubated with primary antibody (anti-RAGE IgG). Where indicated, purified bovine 35-kDa AGE-binding protein (50 μg/ml) was added to mixtures simultaneously with primary antibody. Sites of primary antibody binding were visualized using the peroxidase method according to the manufacturer's instructions (Amersham Corp.), and by using an affinity purified ^{125}I-anti-guinea pig IgG (Sigma). The approximate molecular masses of protein bands were determined by comparison with standards run simultaneously (Rainbow Standard, Amersham Corp/): phosphorylase *b*, 97.4 kDa; bovine serum albumin 69 kDa; ovalbumin, 46 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 21.5 kDa; and lysozyme, 14.3 kDa.

Radioligand Binding Studies—Radioligand binding studies were performed by growing 293 cells transfected with the pD5 plasmid containing the bovine RAGE cDNA or pD5 mock-transfected control to confluence in 96 wells previously coated with 2.5 μg/cm^2 poly-D-lysine (Sigma). Cells were washed three times with Hank's balanced salt solution, pH 7.4, binding buffer (minimal essential medium containing 1% bovine serum albumin (Sigma), 0.05 ml/well) was added along with the indicated amount of ^{125}I-AGE albumin alone or in the presence of at least a 20-fold molar excess of unlabeled AGE albumin. (^{125}I-AGE-albumin and unlabeled AGE-albumin were prepared as described in Ref. 7). Wells were incubated for 2 h at 4 °C, binding was terminated by five washes in ice-cold Hank's buffered salt solution (0.2 ml/wash), and then 0.1 ml elution buffer (minimal essential medium containing 1% Triton X-100) was added for 5 min at 37 °C. The contents of the well were then aspirated and counted in a Rackgamma counter. For studies employing antibodies to AGE-binding proteins and nonimmune IgG, each prepared from guinea pig sera, the IgG, at the indicated concentration, was preincubated with cells for 15 min at 4 °C, and then a radioligand binding assay was performed as above.

RESULTS

Tryptic Map of 35-kDa AGE-binding Protein—The purified bovine 35-kDa AGE-binding protein was subjected to tryptic digestion and chromatography on HPLC reversed-phase in order to obtain peptides for internal sequence analysis (Fig. 1). The elution profile of tryptic fragments from the reversed-phase column was different under nonreducing and reducing conditions, suggesting the existence of internal disulfide bonds. Furthermore, each of the protein sequences obtained was consistent with the hypothesis that the 35-kDa AGE-binding protein was unique, as was suggested by the amino-terminal sequence (7).

RAGE cDNA Cloning—The bovine lung λgt11 cDNA li-

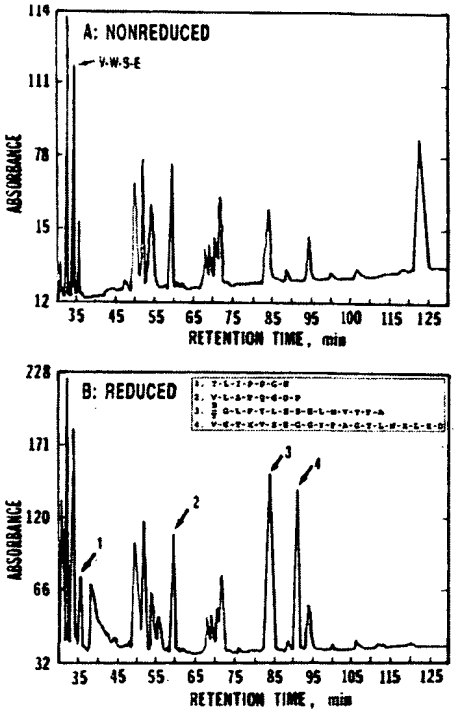

FIG. 1. **HPLC reversed-phase chromatography of tryptic digest and protein sequence of fragments from bovine 35-kDa AGE receptor.** The purified 35-kDa AGE-binding protein was incubated with trypsin, and the reaction mixture was chromatographed on reversed-phase HPLC under nonreduced (*A*) or reduced (*B*) conditions. The elution profile shows adsorption at OD_{215} nm, and peaks were sequenced as indicated (in the *lower panel*, the *numbers* correspond to the peaks sequenced). Amino acids are denoted by the single letter code: Ala (*A*), Cys (*C*), Asp (*D*), Glu (*E*), Phe (*F*), Gly (*G*), His (*H*), Ile (*I*), Lys (*K*), Leu (*L*), Met (*M*), Asn (*N*), Pro (*P*), Gln (*Q*), Arg (*R*), Ser (*S*), Thr (*T*), Val (*V*), Trp (*W*), and Tyr (*Y*). *X* is an amino acid residue not identified at that position.

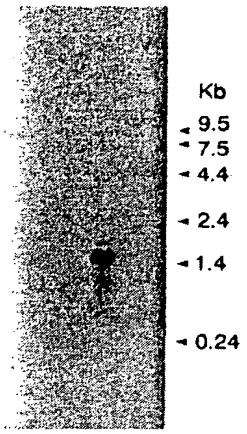

FIG. 2. **Northern of bovine lung total RNA.** 25 μg of bovine lung total RNA and 5 μg of RNA markers were each heated at 55 °C in denaturation buffer for 10 min and then loaded on a 1.2% formaldehyde agarose gel. After electrophoresis, the RNA was transferred to nylon support as described in text and was hybridized 16 h with the ^{32}P-labeled *Eco*RI fragment of the bovine RAGE clone. The blot was then washed (0.5 × SSC at 55 °C final) and exposed overnight with an enhancing screen. The *numbers* to the *right* indicate the position for each RNA marker, in kilobases.

brary was plated and screened using the NH_2-terminal sequence probe. Approximately one in 12,000 clones was positive, and 10 such clones were chosen for additional characterization. These clones exhibited similar restriction patterns (data not shown) suggesting they were likely to be closely related. One clone, containing a ~1,400 base pair (bp) insert, was chosen as a probe for Northern analysis of bovine lung RNA (Fig. 2). A single transcript of about 1,500 nucleotides was observed, suggesting that the cDNA isolated was nearly full-length. The DNA sequence of this clone was determined (Fig. 3, *left panel*). The cDNA is 1440 bp and appears polyadenylated despite containing a modified polyadenylation signal of AGTAAA *versus* the consensus AATAAA (17) starting at −20 bp from the adenylation site. The cDNA encodes a precursor protein of 416 amino acids and includes all five of the peptide sequences obtained from protein sequencing of tryptic peptides (Fig. 1) as well as the amino-terminal sequence (7). The protein includes a putative signal sequence of 22 amino acids and an extracellular domain of 332 amino acids. The latter domain contains 6 cysteines, evenly spaced with 42–64 amino acid residues between them, and two probable *N*-linked glycosylation sites located near the amino terminus (at amino acid positions 3 and 58 of the mature protein). There is also a putative transmembrane region of 19 hydrophobic amino acids followed by a highly charged intracellular domain of 43 amino acids.

The human counterpart of bovine RAGE was obtained from a human lung cDNA library using the bovine clone as a probe. The DNA sequence of this partial clone is presented in Fig. 3, at right. This human cDNA contains 1406 bp and appears to encode the entire mature protein of 404 amino acids. Like the bovine form, human RAGE protein appears to contain a single membrane-spanning domain of 19 amino acids separating the protein into an extracellular domain of 321 amino acids and an intracellular domain of 41 amino acids. The protein appears to contain a signal sequence at least as long as that of bovine RAGE, although in this clone the initiating methionine is not present, presumably because the clone is truncated. Most, if not all of the signal sequence is present, however, and is apparently cleaved after glycine 22 based on computer analysis using the Sigcleave program. The putative polyadenylation sequence ATTAAA, located −23 bp from the site of adenylation differs from the bovine sequence, AGTAAA, but it is curious in that it also differs from the consensus sequence, AATAAA. An alignment of the deduced protein sequences for the human and bovine clones is shown in Fig. 4. They share an overall identity of 83.6% and are 90.8% similar. The bovine sequence contains an 11-amino-acid insertion with respect to the human starting at tryptophan 230.

Initial database searches conducted using the bovine and human RAGE peptide sequences suggested RAGE is a member of the immunoglobulin superfamily of sequences. A profile of RAGE was constructed using the human and bovine RAGE peptide sequences and was used to search Protein Identification Resource and SwissProt. The top match in these searches was the MUC 18 sequence, with a ZScore of 5.64. MUC 18 and human RAGE share 24.8% identity and 48.5% similarity over a 612-residue alignment (including gaps). Bovine RAGE shares 24.3% identity and 46.8% similarity with MUC 18 over a 611-residue alignment. MUC 18 is an immunoglobulin-like superfamily member glycoprotein used as a marker of tumor progression in melanoma (30). Both RAGE and MUC 18 share sequence similarity to the neural cell adhesion molecules (31). Bovine RAGE also shares homology with the B-cell activation marker CD20 (33) with 36% identity when comparing the carboxyl-terminal 48 residues of RAGE to the cytoplasmic domain of CD20. Further analysis of RAGE has shown that it has three possible immunoglobulin-like do-

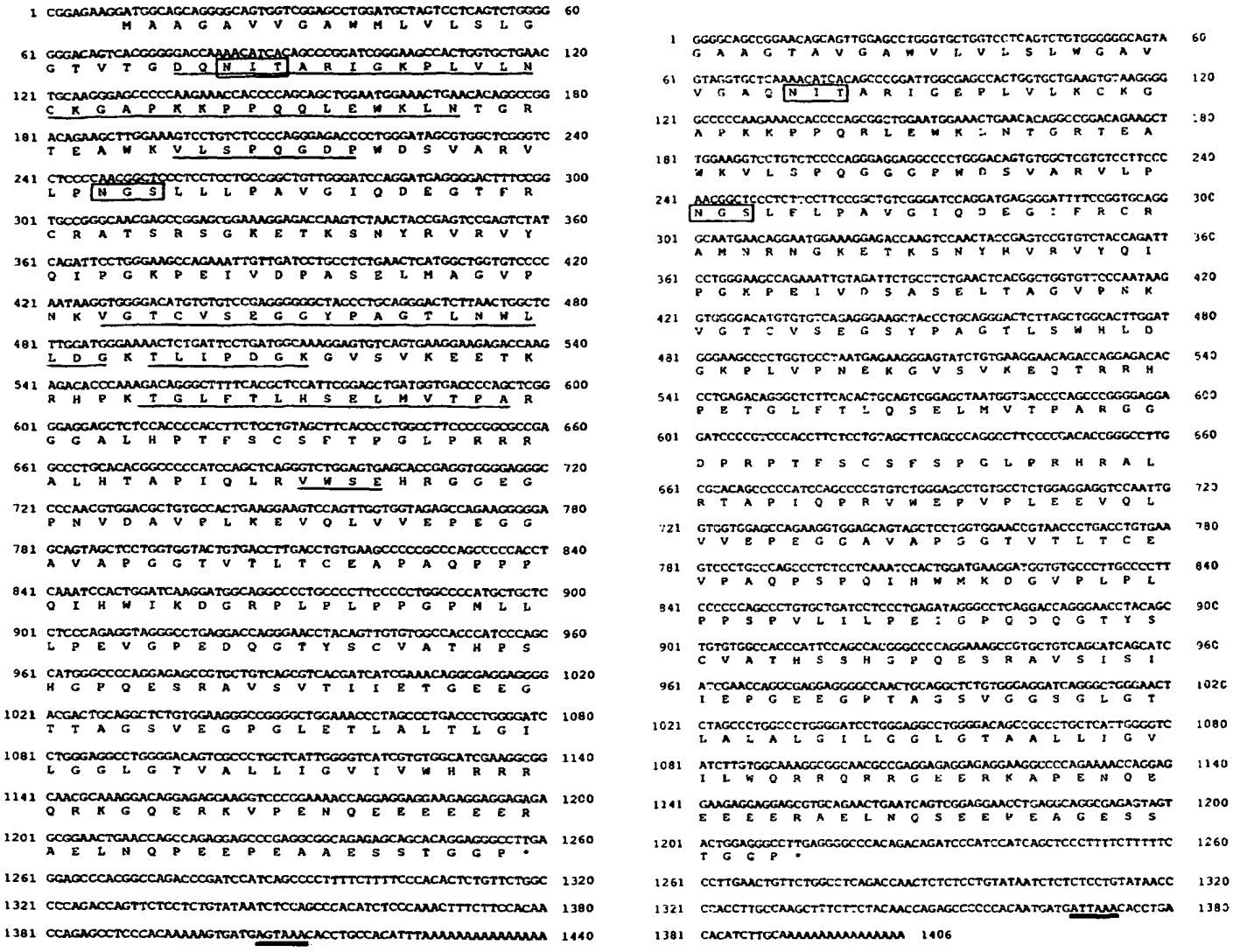

FIG. 3. **Nucleotide and amino acid sequence of bovine and human RAGE.** The bovine (*left*) and human (*right*) genes were sequenced by the dideoxy chain termination method as described under "Materials and Methods." Potential *N*-linked glycosylation sites are indicated by *boxed sequences*, the putative polyadenylation sites are shown with *bold underlining*, and sequences matching the sequenced bovine peptides are indicated by *light underlining*. The following amino acid residues from the *underlined* peptide sequences were not determined by the protein sequencing: all Cys (*C*) and Trp (*W*), Asn^{25} (N^{25}) and Glu^{50} (E^{50}).

mains, each with a set of conserved cysteine residues. The location of these immunoglobulin-like domains within the RAGE molecule is depicted in Fig. 5. The first domain has characteristics of a V-like domain and includes the two potential *N*-linked glycosylation sites. The two remaining domains have properties of C-like domains. A Motif search identifies an immunoglobulin/major histocompatibility complex signature sequence at position 309 in the bovine sequence (299 in the human form). This sequence, YSCVATH, is usually found around the COOH-terminal cysteine involved in the intradomain disulfide bond. The consensus pattern is (F, Y)xCx(V, A)xH. This pattern is indicative of an IG constant type domain.

Expression of Bovine RAGE in 293 Cells—Indirect immunofluorescence on nonpermeabilized, pD5-RAGE transiently transfected 293 cells showed surface expression of the RAGE protein, whereas the mock-transfected cells were negative (Fig. 6, *panels I versus II*). In contrast, 293 cells transfected with the RAGE cDNA or mock-transfected controls did not express material immunoreactive with antibody to the other AGE-binding protein, a lactoferrin-like polypeptide (7, data not shown).

In parallel with immunofluorescence staining of the cell surface, Western blotting with the anti-AGE-binding protein IgG demonstrated its expression in 293 cells transfected with the RAGE cDNA (Fig. 7). 293 cells transfected with the RAGE cDNA were extracted with detergent and the protein was concentrated by adsorption to hydroxylapatite (the latter resin strongly adsorbs the 35-kDa AGE-binding protein) (7). The hydroxylapatite eluate was then subjected to SDS-PAGE. Compared with a preparation from the same number of mock-transfected 293 cells, only the 293 cells transfected with the RAGE cDNA demonstrated significant amounts of protein visible by Coomassie staining on 10% SDS-PAGE of the hydroxylapatite eluate (Fig. 7, *lanes 1–2*). The pattern of protein bands in the transfected 293 cells was clearly more

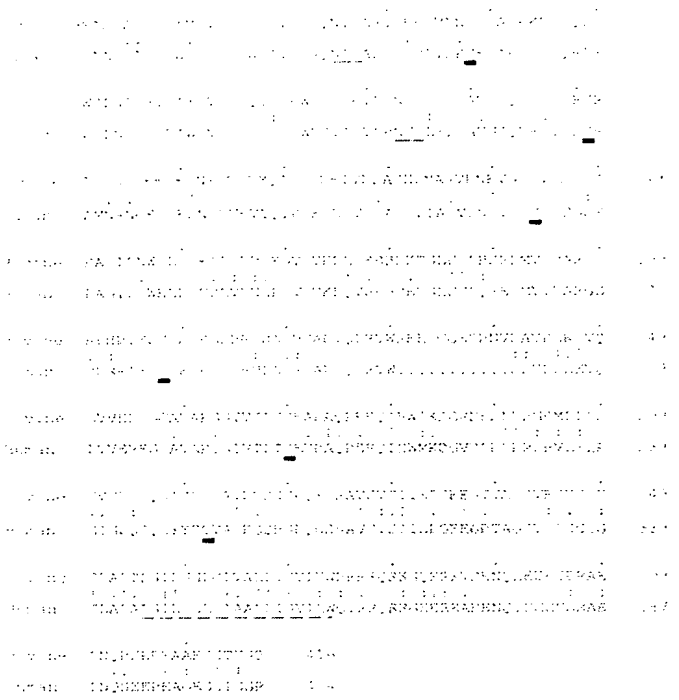

FIG. 4. **Protein sequence alignment of bovine and human RAGE.** Sequences were deduced from translation of the cDNAs, and alignments were made using the GAP program of GCG. *Vertical lines* show sequence identity. *Vertical rows of dots* indicate threshold similarity. Gaps, indicated by *dots* were introduced to produce optimal alignment. *Underlining* indicates conserved cysteines (*bold underline*), *N*-linked glycosylation sites (*light underline*), and transmembrane domain (*dashed underline*).

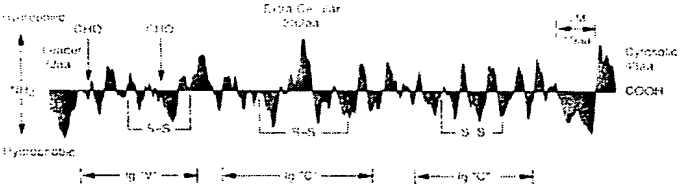

FIG. 5. **Hydrophilicity plot of bovine RAGE.** The hydrophilicity profile was generated from the Hopp and Woods program of intelligenetics. NH_2, amino terminus; *COOH*, carboxyl-terminus; *CHO*, *N*-linked glycosylation sites; *TM*, transmembrane domain; *S-S*, disulfide-linked cysteine residues; *IgV* and *IgC*, immunoglobulin-like variable and constant domains, respectively.

complex than that observed when the purified bovine 35-kDa AGE-binding protein obtained from lung tissue was subjected to SDS-PAGE (Fig. 7, *lane 3*).

Western blot analysis of extracts from transfected 293 cells was performed to permit identification of RAGE-immunoreactive material. Extracts from mock-transfected 293 cells showed no bands with the anti-AGE-binding protein IgG (Fig. 7, *lane 4*), while the RAGE-transfected cells showed a major band of ~50 kDa (Fig. 7, *lane 5*). Prolonged exposure of the blot to film demonstrated several additional fainter bands with M_r values of 30,000–35,000, 55,000, and 80,000. This approximated the pattern of bands observed in the Coomassie-stained gel of the hydroxylapatite eluate of the RAGE-transfected cells (Fig. 7, *lane 2*). Western blotting of the purified lung 35-kDa AGE-binding protein with anti-35-kDa antibody demonstrated only a single band (Fig. 7, *lane 6*). To be certain that all immunoreactive bands observed in extracts derived from the transfectants were due to epitopes derived

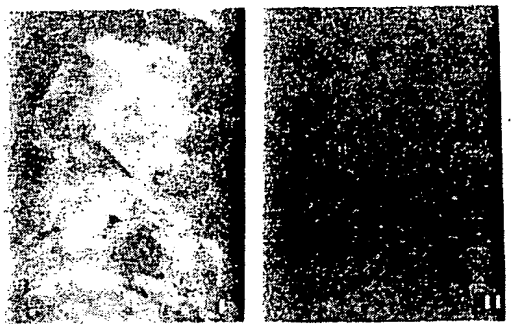

FIG. 6. **Indirect immunofluorescence of bovine RAGE-transfected 293 cells using anti-RAGE IgG.** 293 cells transfected with the cDNA for RAGE (*panel I*) or mock-transfected controls (*panel II*) were prepared for immunofluorescence using anti-35-kDa AGE-binding protein IgG as described in the text.

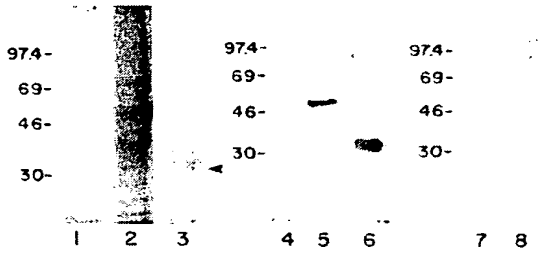

FIG. 7. **Western blotting of extracts of transfected 293 cells with anti-35-kDa AGE-binding protein IgG.** 293 cells transfected with the cDNA for RAGE and mock-transfected controls (10^5 cells in each case) were extracted in detergent-containing buffer and processed for nonreduced SDS-PAGE (10%) as described in the text, including adsorption to hydroxylapatite, precipitation of the eluate in trichloroacetic acid, and solubilization of the pellet in nonreduced SDS gel sample buffer. Samples from 293 cells transfected with the cDNA for RAGE and mock-transfected controls were treated identically, and the same volume of sample was applied to each lane of the gel. *Lanes 1* and *2* demonstrate proteins visualized by Coomassie Blue staining of mock-transfected control 293 cells and 293 cells transfected with the RAGE cDNA, respectively. *Lane 3* demonstrates the band obtained on Coomassie Blue staining with purified 35-kDa AGE-binding protein (3 μg). An *arrow* designates migration of the single band in *lane 3*. Western blotting of these samples was performed, and blots were stained with anti-35-kDa AGE-binding protein IgG (50 μg/ml) in *lanes 4* and *5* (mock-transfected control 293 cells and 293 cells transfected with RAGE, respectively). *Lane 6* demonstrates Western blotting with purified 35-kDa AGE-binding protein (3 μg). Western blotting of 293 cells transfected with the RAGE cDNA and purified 35-kDa AGE-binding protein (3 μg) was also performed in the presence of purified soluble RAGE (50 μg/ml; the latter was added during the incubation with primary antibody) (*lanes 7* and *8*, respectively).

from the AGE-binding protein. Western blotting was repeated in the presence of an excess of soluble purified bovine 35-kDa AGE-binding protein (added during the incubation of blots with the anti-35-kDa AGE-binding protein IgG). All bands disappeared from the 293 cells transfected with RAGE cDNA and from the purified 35-kDa AGE-binding protein (Fig. 7, *lanes 7* and *8*, respectively).

In view of the expression of RAGE protein by transfected 293 cells, we considered whether recombinant RAGE could mediate cell surface binding of AGEs. Radioligand binding studies with ^{125}I-AGE-albumin demonstrated specific, dose-dependent, and saturable binding on 293 cells transfected with RAGE with K_d ~100 nM (Fig. 8*A*), which is similar to what is observed on cultured endothelial cells (6–7) and mononuclear phagocytes (8). Mock-transfected 293 cells showed no specific binding of ^{125}I-AGE-albumin. The central

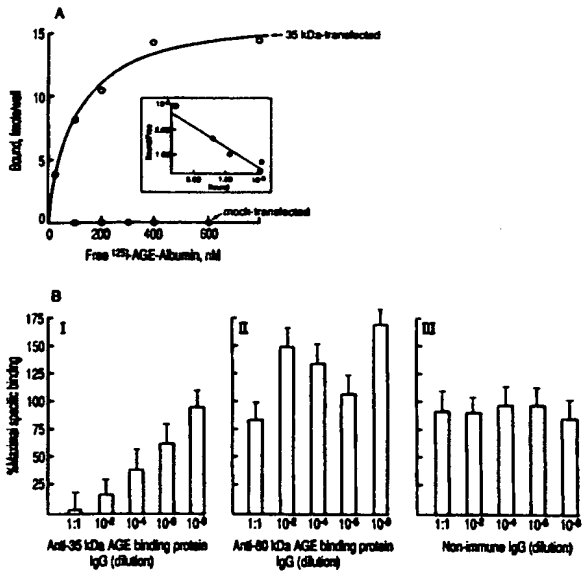

FIG. 8. **Binding of ^{125}I-AGE-albumin by transfected 293 cells.** *A*, dose response. A radioligand binding assay was performed on 293 cells transfected with the cDNA for RAGE (*open circles*) or mock-transfected 293 controls (*closed circles*) by adding the indicated concentrations of ^{125}I-AGE-albumin alone or in the presence of unlabeled AGE albumin (20-fold molar excess). After the incubation period, cultures were washed, and eluted cell-bound radioactivity was counted as described in the text. Each point is the mean of quadruplicate determinations, and the *inset* shows Scatchard analysis of the same data. Parameters of binding are $K_d = 100 \pm 20$ nM (value ± S.E.) and $n = 17 \pm 1$ fmol/well. *B*, effect of anti-35 and antilactoferrin-like AGE-binding protein IgG and nonimmune IgG on the binding of ^{125}I-AGE albumin to 293 cells transfected with the cDNA for RAGE. 293 cells transfected with the cDNA for RAGE were preincubated with either anti-35-kDa AGE-binding protein IgG (1:1 dilution, 3.7 mg/ml, *BI*), anti-lactoferrin-like (80 kDa) AGE-binding protein IgG (1:1 dilution, 2.7 mg/ml, *BII*), or nonimmune IgG (1:1 dilution, 3.7 mg/ml, *BIII*) for 15 min at 4 °C, and subsequently a radioligand binding assay was performed by adding ^{125}I-AGE-albumin (100 nM) alone or in the presence of a 20-fold molar excess of unlabeled AGE albumin at 4 °C for 2 h. Bound radioactivity was determined as described in the text. Each experiment employed quadruplicate determinations, and the experiment was repeated five times. The mean and S.E. are shown.

role of RAGE in mediating AGE-cellular interaction in this context was shown by the inhibitory effect of anti-35-kDa AGE-binding protein IgG (Fig. 8*B*). In contrast, antibody to the lactoferrin-like AGE-binding protein IgG and nonimmune IgG had no inhibitory effect (Fig. 8, *C* and *D*).

DISCUSSION

Binding of AGE-modified proteins to the endothelial cell involves two surface-associated polypeptides: a lactoferrin-like AGE-binding protein (7) and a 35-kDa polypeptide, which has been characterized in the current study. Molecular cloning of this AGE-binding protein has shown it to be a new member of the immunoglobulin superfamily of receptors, leading us to tentatively assign it the name RAGE. Consistent with our previous findings that the binding of AGEs to RAGE required no serum cofactors, 293 cells transfected only with the cDNA for RAGE-bound ^{125}I-AGE-albumin in a dose-dependent manner in serum-free media (these cells did not demonstrate any immunoreactivity with the lactoferrin-like AGE-binding protein). The K_d for binding of ^{125}I-AGE-albumin to these cells, ~100 nM, was close to that observed for binding of AGEs to endothelial cells and mononuclear phagocytes (6–9). Binding of radioiodinated AGE ligand to 293 cells transfected with RAGE cDNA was blocked by antibodies to RAGE, and was accompanied by evidence of expression of the receptor, based on Western blotting. The receptor extracted from these 293 cells demonstrated a major immunoreactive band at M_r ~50,000, as well as several other bands with M_r values as low as 30,000–40,000. Since the appearance of all of these bands was blocked by the addition of purified RAGE, it is most likely that their appearance in the RAGE-transfected cells reflected post-translational processing. In this context, the calculated molecular mass of the recombinant protein minus the signal sequence is 42,141 Da, to which about 5,000 Da must be added to account for the two *N*-linked polysaccharides. Thus, there is probably extensive processing of RAGE following its translation.

The 35 kDa form of RAGE isolated from acetone powder of bovine lung is likely to be one of these post-translationally processed forms, potentially the product of proteolytic cleavage *in situ* or during the purification procedure. Although definitive proof for this hypothesis will require a detailed comparison of tryptic maps of RAGE derived from lung tissue with purified material obtained from transfected cells (the latter will require much larger amounts than are currently available), it is relevant to note that all of the tryptic fragments sequenced thus far from the purified lung material were derived from the amino-terminal half of the protein (Fig. 1). Thus, it is possible that the 35 kDa form of RAGE is due to proteolytic processing at the carboxyl terminus. Furthermore, in view of the presence of RAGE in a form migrating with M_r ~50,000 in the transfected cells, it is tempting to speculate that this polypeptide may be related to the 60-kDa protein which binds AGE-albumin identified by Yang *et al.* (8). However, the sequence published by these authors bears no similarity to human or bovine RAGE.

These observations lead us to speculate that there are likely to be several types of AGE-binding proteins potentially recognizing different classes of AGE ligands or activating distinct cellular processes following formation of the ligand-receptor complex. Skolnik *et al.* (9) recently identified AGE-binding proteins of 30, 40, and 50 kDa, based on ligand blotting of renal tissue with radioiodinated AGE-albumin. Although the latter may represent unique proteins, it is possible that these AGE binding species are related to RAGE based on the presence of similarly migrating bands on Western blots of RAGE-transfected cells (Fig. 7) and partially processed lung extracts (data not shown). In this context, our pilot studies have shown positive immunostaining of renal tissue with anti-AGE-binding protein antibody.

Although initially we speculated that cellular AGE receptors might resemble other scavenger receptors, such as receptors which facilitate uptake of acetylated low density lipoprotein or asialoglycoprotein (23–26), RAGE is a member of the immunoglobulin superfamily. The immunoglobulin superfamily comprises, in addition to the immunoglobulins, a broad array of cell surface receptors and adhesion molecules (see Ref. 32, for a recent review). The overall architecture of RAGE is consistent with either of these two functions. RAGE has three domains, one most similar to the variable domain set and two that resemble the constant C2 set. RAGE also has a typical membrane-spanning sequence and an acidic carboxyl-terminal cytoplasmic tail. The cytoplasmic tail has reasonable similarity to the cytoplasmic tail of the CD20. This suggests that RAGE may subserve functions beyond binding and subsequent uptake of AGEs. Two lines of evidence in this regard implicate RAGE as a cell adhesion molecule or growth factor receptor. Pilot studies have demonstrated that RAGE contributes to the enhanced adherence of diabetic red cells to

endothelium (27). In addition, AGEs can stimulate endothelial proliferation (28), and preliminary experiments have shown that antibodies to RAGE also directly stimulate endothelial growth. This suggests the hypothesis that AGEs are accidental and potentially pathogenic ligands for this receptor and indicate that an important challenge for future studies will be to identify the natural ligand of RAGE, potentially a growth factor or cell surface ligand.

The results presented in this and the previous study (7) serve as a starting point for experiments to elucidate functions of RAGE and AGEs in pathophysiology by providing essential reagents for detection and expression of RAGE in cells and tissues. Previous work demonstrating that AGEs modulate multiple properties of endothelial cells, including permeability, growth, and thrombogenicity, and of mononuclear phagocytes (6, 26), such as cell migration and production of cytokines-growth factors (8, 9, 11), suggests that exploration of biologic functions of RAGE will be a fruitful undertaking.

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